(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
6 September 2002 (06.09.2002)

PCT

(10) International Publication Number
WO 02/068627 A2

(51) International Patent Classification[7]: C12N 15/00

(21) International Application Number: PCT/US02/05280

(22) International Filing Date: 22 February 2002 (22.02.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/270,885 23 February 2001 (23.02.2001) US

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

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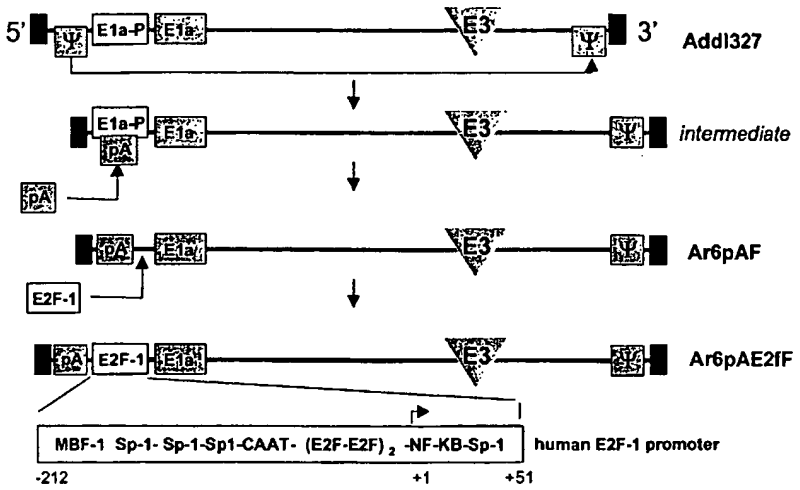
(54) Title: NOVEL VECTOR CONSTRUCTS

Schematic diagram of Ar6pAF and Ar6pAE2fF vectors



(57) Abstract: The present invention provides novel viral vectors and methods useful for the minimization of leaky gene expression, and, in particular, of nonspecific transcriptional read-through of genes. Such constructs may be obtained by the insertion of an insulating sequence into a vector construct, such as for example a termination signal sequence upstream of the transcription initiation site of the respective transcription unit.

WO 02/068627 A2

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

NOVEL VECTOR CONSTRUCTS

This application claims the benefit of US Patent Application No. 60/270,885, filed February 23, 2001, which is herein incorporated by reference.

FIELD OF THE INVENTION

The present invention generally relates to recombinant DNA technology and the regulation of gene expression. Specifically, it relates to viral vectors that provide for controlled gene expression in the field of gene therapy.

BACKGROUND OF THE INVENTION

Gene expression in prokaryotic and eukaryotic cells is regulated on the transcriptional and translational levels. For trancription to occur, RNA synthesis is catalyzed by the enzyme RNA polymerase. Trancription starts when RNA polymerase binds to a special region, the promoter, at the start of the gene. The promoter usually precedes the first base pair that is transcribed into RNA, the startpoint. From this point, RNA polymerase moves along the template, synthesizing RNA, until it reaches the termination sequence. This action defines a transcription unit on the DNA molecule that extends from the transcription initiation site (startpoint) to the terminator.

Regulation of gene expression on the transcriptional level occurs by various mechanisms. Gene expression is controlled by particular regulatory sequences, such a promoters and enhancers, to which cellular factors may bind and thereby alter the expression rate of the adjacent gene. Such cellular factors include, for example, so-called transcription factors, which are proteins required for the recognition by RNA polymerases of specific binding sequences in genes.

Certain applications of recombinant DNA technology require that a gene be tightly regulated by its promoter, ie. that the transcription level of the gene is not dependent on any cis-acting elements other than the promoter itself. For example, in the context of gene therapy, the tissue selectivity of a viral vector administered for a therapeutical purpose may rely on the specific regulation of a gene which, therefore, should be tightly regulated by its promoter. One such gene therapy approach is directed to cancer and utilizes so-called "oncolytic adenoviral

vectors." (See, for example, US 5,998,205 (Hallenbeck et al.) Oncolytic adenoviral vectors are adenoviral vectors that are tumor-specific and replication competent after infection of the target cell in the organism. In this approach, a gene that is essential for the replication of an adenoviral vector is regulated by a tissue-specific promoter and thereby provides for tissue-specificity of the replication of the vector. Thus, in this approach, the adenoviral vectors will specifically replicate and lyse tumor cells if the gene that is essential for replication is exclusively under the control of a promoter that is tumor-specific, and is not induced by additional genetic elements that are not tissue-specific.

It is an object of the present invention to provide viral vectors, such as, for example, adenoviral vectors, that allow for the specific and tight regulation of a gene of interest within the viral vector. In the context of oncolytic adenoviral vectors, it is a further object of the present invention to provide for vectors with a high degree of tissue specificity.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Cleavage and polyadenylation process for the SV40 early poly(A) site.

Figure 2: E1A transcription control region.

Figure 3: Sequence of Ar6pAE2fF from left and right ends of viral DNA. Regions of Ar6pAE2fF confirmed by DNA sequencing. Panel A. Regions in first 1802 nucleotides are ITR (nucleotides 1-103), poly-adenylation signal (nucleotides 116-261), human E2F-1 promoter (nucleotides 283-555), E1a gene (nucleotides 574-1647) and a portion of the E1b gene (nucleotides 1648-1802). Panel B. Regions in the last 531 nucleotides are the PacI restriction site (nucleotides 33967-33974) (underlined), the packaging signal (nucleotides 34020-34217 and the ITR (34310-34412).

Figure 4: Sequence of Ar6F from left end of viral DNA. The first 660 nucleotides at the left end of Ar6F. The ITR (nucleotides 1-103), a multiple cloning site (MCS) (nucleotides 104-134) and a portion of the E1a gene (nucleotides 135-660) are shown.

Figure 5: Sequence of Ar6pAF from left end of viral DNA. The first 660 nucleotides at the left end of Ar6pAF. The ITR (nucleotides 1-103), the SV40 early polyA signal (nucleotides 104-134) and a portion of the E1a gene (nucleotides 298-660) are shown.

Figure 6: Schematic diagram of Ar6pAF and Ar6pAE2fF vectors. The backbone adenoviral sequences are derived from the pAr6pAF and pAr6pAE2fF infectious plasmids. The intermediate vector backbone adenoviral sequences are derived from Addl327, an E3-deleted adenovirus type 5, in which the packaging signal is located immediately upstream of the right ITR. The Ar6pAF vector backbone is deleted in the E1a promoter, and the SV-40 poly(A) signal is inserted after the left ITR. The Ar6pAE2fF vector backbone contains, after the SV-40 poly(A) signal sequences, the E2F-1 promoter (bp-212 to +51), a DNA segment of four intact E2F, one NF-kB and four Sp1 consensus sequences.

Figure 7: Comparison of body weight change after administration of vectors Addl327, AvE1aPA09lxi, Ar6F, Ar6pAF, Addl312.

Figure 8: Backbones of vectors Addl327, AvE1a09i, AvPAE1a09i, Ar6F, Ar6pAF, Addl312.

SUMMARY OF THE INVENTION

The present invention provides novel viral vectors that exhibit improved regulation of one or more genes within the viral vector. In such vectors, transcription is tightly controlled by its promoter and is essentially independent of interfering genetic elements, such as, for example, cis-acting elements located in the viral vector construct itself.

Accordingly, in one aspect, the present invention provides a viral vector, which has at least one interfering genetic element, comprising at least one transcription unit, wherein at least one insulating sequence is located 5' to the transcription initiation site of said transcription unit and 3' to said interfering genetic element.

In another aspect of the invention a viral vector particle comprising the viral vector of the invention is provided.

In a further aspect of the invention, a eukaryotic cell transfected with the viral vector particle of the invention is provided.

In yet another aspect of the invention, a method of reducing the transcription level of a transcription unit in a viral vector caused by an interfering genetic element is provided, comprising the steps of identifying a suitable insulating sequence and inserting said insulating sequence into said viral vector 5' to the transcription initiation site of said transcription unit.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides novel viral vectors and, in particular, novel adenoviral vectors. Such vectors may be obtained by the insertion of an insulating sequence into a viral vector, such as, for example, the insertion of a termination signal sequence upstream of the startpoint (transcription initiation site) of the transcription unit to be shielded from non-specific transcriptional read-through. The viral vectors of the invention show a reduced amount of "leaky expression" of the gene of interest as compared to viral vectors which do not include the insulating sequence.

Generally, protein expression involves the transcription of a gene into mRNA and the translation of this mRNA into a polypeptide. Depending on the context in which used, the term "expression" may refer to the production of RNA, protein or both. The present invention is primarily concerned with the process of transcription into mRNA and its regulation.

Leaky expression is gene expression which is independent of the promoter directly upstream of the gene. In the context of gene therapy, leaky gene expression may reduce the specificity of certain therapeutic approaches. For example, the delivery of a heterologous gene may be dependent on the activation of a tissue-specific promoter driving said gene in a particular cellular environment, thereby avoiding that the gene is expressed in tissues which do not produce factors that activate the tissue-specific promoter. Such an approach will be less specific if leaky expression of the heterologous gene occurs.

The present invention now provides a method to shield a transcription unit from the unwanted regulatory influence of an interfering genetic element in a viral vector.

A transcription unit within the meaning of the invention may include one or more genes. Trancription starts when RNA polymerase binds to a special region, the promoter, at the start of the gene (the startpoint or transcription initiation site). The startpoint is the first base pair that is transcribed into RNA. From this point, RNA polymerase moves along the template, synthesizing RNA, until it reaches the termination sequence. This action defines a transcription unit that extends from the transcription initiation site to the terminator. Generally, the first nucleotide in the transcript is defined as position +1 of the transcription unit. The nucleotide immediately preceding this on the corresponding DNA strand is defined as position -1.

A transcription unit may be "operably linked" to a "regulatory element". A nucleic acid sequence is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For instance, a regulatory element is operably linked to a transcription unit if it affects the transcription of said transcription unit. Operably linked DNA sequences are typically contiguous. However, since enhancers generally function when separated from the promoter by several kilobases and intronic sequences may be of variable lengths, some nucleic acid sequences may be operably linked but not contiguous. As used herein, the term "regulatory element" refers to a genetic element which controls some aspect of the transcription of nucleic acid sequences. Examples of regulatory elements are promoters, enhancers, polyadenylation signals, termination signals, etc.

In one aspect, the present invention provides a viral vector having at least one interfering genetic element and comprising at least one transcription unit, wherein at least one insulating sequence is located 5' to the transcription initiation site of said transcription unit and 3' to said interfering genetic element.

As used herein, the term "viral vector" is used according to its art-recognized meaning. It refers to a nucleic acid vector construct which includes at least one element of viral origin and may be packaged into a viral vector particle. The viral vector particles may be utilized for the purpose of transferring DNA into cells either in vitro or in vivo. Viral vector particles that have been commonly used for the latter purpose include particles based on retroviruses (including lentiviruses), adenoviruses, parvoviruses (such as adeno-associated viruses), and herpes viruses.

The term "interfering genetic element" is to be understood in a broad sense. Interfering genetic elements may display unwanted enhancer or promoter activity in relation to a transcription unit. In particular, an interfering genetic element of the invention may have an influence on the activity of the promoter which is directly adjacent and upstream of the gene in question. Interfering genetic elements may in particular be interfering promoters or enhancers. Enhancer or promoter activity is to be understood as any activity that increases the transcription level, i.e. the detectable amount of primary RNA transcript from the transcription unit in question. Accordingly, interfering genetic elements can be assayed by measuring transcription of any downstream gene, for example, by RT-PCR or Northern detection systems.

An interfering genetic element may have a important function which should be preserved in a viral vector. For example, in the field of adenoviral vector construction, the ITRs are critical for adenoviral DNA replication. Furthermore, sequences downstream of the left ITR are necessary for proper packaging of the viral genome. Thus, when constructing viral vectors, it may not always be possible to identify and/or delete all interfering genetic elements which display enhancer or promoter activity in relation to a transcription unit.

The term "promoter" is used according to its art-recognized meaning. It is intended to mean the DNA region, usually upstream to the coding sequence of a gene, which binds RNA polymerase and directs the enzyme to the correct transcriptional start site (transcription initiation site). Promoters are located immediately upstream (5') from the start site of transcription. Promoter sequences are required for accurate and efficient initiation of transcription. A typical promoter includes an AT-rich region called a TATA box, which is typically located approximately 30 base pairs 5' from the transcription initiation site.

The term "enhancer" is used according to its art-recognized meaning. It is intended to mean a sequence found in eukaryotes and certain eukaryotic viruses which can increase transcription from a gene when located up to several kilobases from that gene. These sequences usually act as enhancers when on the 5' side (upstream) of the gene in question. However, some enhancers are active when placed on the 3' side (downstream) of the gene. In some cases, enhancer elements can activate transcription from a gene with no known promoter. Thus, enhancers increase the rate of transcription from the promoter sequence. It

is predominantly the interaction between sequence-specific transcriptional factors with the promoter and enhancer sequences that enable mammalian cells to achieve tissue-specific gene expression. The presence of these transcriptional protein factors bound to the promoter and enhancers enable other components of the transcriptional machinery, including RNA polymerase, to initiate transcription with tissue-specific selectivity and accuracy.

In a preferred embodiment of the viral vector, the insulating sequence is located directly upstream of the regulatory element to be shielded from the interfering genetic element. Dependent on the size of the regulatory element, preferredly, the insulating sequence is located no more than 3000 nucleotides upstream (5') to the transcription initiation site of the transcription unit, more preferredly, no more than 500, 300 or even 200 nucleotides. However, if a minimal promoter is used, the insulating sequence may be located no more than 17 nucleotides 5' to the transcription initiation site of the transcription unit. Preferredly, the insulating sequence is located upstream of the first transcription unit from the 5' end of the viral vector. In particular, the insulating sequence may preferredly be located upstream of the first transcription unit (as seen from the 5' end of the viral vector) which encodes a gene which is essential for replication in the respective vector. For example, if the viral vector is an adenoviral vector, the insulating sequence is preferredly located upstream of the E1a transcription unit. It is to be understood that in the context of adenoviral vector the terms " 5' " and "upstream" are understood to correspond to the left ITR of the adenoviral vector.

Insulating sequences are segments of DNA that serve to isolate a gene by blocking interactions between e.g. enhancers on one side of the insulating sequence from the promoters of neighboring genes. For the purposes of the present invention, the term is to be understood in a broad functional sense. The defining characteristic of an insulating sequence within the meaning of the invention is its ability to insulate or protect a defined transcription unit which is operably linked to a regulatory element from the influence of an upstream interfering genetic element when located between the interfering genetic element and the regulatory sequence of the transcription unit to be insulated. Preferredly, insulating sequences of the invention are segments of DNA that have been isolated from their genetic source. The insulating sequence sequence may then be inserted into the viral vector at a suitable position as further described herein.

In a preferred embodiment of the present invention, the insulating sequence is a termination signal sequence; particularly preferred is a polyadenylation signal sequence. Any polyadenylation signal sequence may be useful for the purposes of the present invention. However, in preferred embodiments of this invention the termination signal sequence is either the SV40 late polyadenylation signal sequence or the SV40 early polyadenylation signal sequence. Preferredly, the termination signal sequence is isolated from its genetic source and inserted into the viral vector at a suitable position as further described herein.

A termination signal sequence within the meaning of the invention may be any genetic element that causes RNA polymerase to terminate transcription. A polyadenylation signal sequence is a recognition region necessary for endonuclease cleavage of an RNA transcript that is followed by the polyadenylation consensus sequence AATAAA. A polyadenylation signal sequence provides a "polyA site", i.e. a site on a RNA transcript to which adenine residues will be added by post-transcriptional polyadenylation. Polyadenylation signal sequences are useful insulating sequences for transcription units within eukaryotes and eukaryotic viruses. Generally, the polyadenylation signal sequence includes a core poly(A) signal which consists of two recognition elements flanking a cleavage-polyadenylation site (Figure 1). Typically, an almost invariant AAUAAA hexamer lies 20 to 50 nucleotides upstream of a more variable element rich in U or GU residues. Cleavage between these two elements is usually on the 3' side of an A residue and in vitro, is mediated by a large, multicomponent protein complex. The complex includes the cleavage and polyadenylation specific factor (CPSF), which binds the AAUAAA motif; the cleavage stimulation factor (CstF), which binds the downstream U-rich element; and two additional cleavage factors (CF I and CF II) that are less well characterized. Also, the poly(A) polymerase must be present in most cases for the cleavage step as well. The choice of a suitable polyadenylation signal sequence will consider the strength of the polyadenylation signal sequence, as completion of polyadenylation process correlates with poly(A) site strength (Chao et al., Molecular and Cellular Biology , Aug. 1999, pp5588-5600). For example, the strong SV40 late poly(A) site is committed to cleavage more rapidly than the weaker SV40 early poly(A) site. The person skilled in the art will consider to choose a stronger polyadenylation signal sequence if a more substantive reduction of nonspecific transcription is required in a particular vector construct.

The present invention also contemplates the use of silencers as insulating sequences. A "silencer" is a DNA region which inhibits transcription initiation by interfering with enhancer activity. The insulating sequence may also be the site of binding of a repressor protein.

In another preferred embodiment of this invention, the vector construct is an adenoviral vector. In an adenoviral vector, the vector construct comprises a genetic element derived from an adenovirus. In a preferred embodiment, it comprises an adenoviral 5'ITR, an adenoviral 3'ITR and an adenoviral packaging signal. The E3 region may or may not be deleted, Accordingly, in one embodiment, the adenoviral vector further comprises a deletion in the E3 region.

An analysis of the characteristics of the nucleotide elements around the adenoviral (Ad5) E1a region indicates that an element containing enhancer like properties lies between –141 and -305 relative to the E1a cap site at +1 (Figure 2). This enhancer element is located very close to a sequence required in cis for packaging of viral DNA. Deletion of the enhancer element reduces both the rate of transcription and steady-state levels of E1a mRNAs in virus-infected cells. The E1a enhancer contains an 11 bp repeat element, which is a critical component of the modulatory sequence (5'-AGGAAGTGACA-3). A 2-3-fold reduction of E1a expression is observed when one copy of the repeat sequence is removed, whereas expression drops 15 to 20 times when both copies are removed (Hearing and Shenk, Cell vol. 33, pp.695-303, July 1983). However, it was found that a deleted mutant can still direct the synthesis of E1a-specific mRNAs, even though it lacks the entire region from –393 to +10 relative to the E1a cap site containing the enhancer and promoters elements. It is not clear which sequences are responsible for this transcription. Accordingly, in the context of adenoviral vectors, the interfering genetic element may be located within the 5'ITR, which is a region necessary for replication of the adenovirus.

In one particular embodiment, the present invention describes a strategy to reduce nonspecific activation of the E1a gene of an adenoviral vector by blocking the read-through transcription from upstream of the E1a promoter. It is found that removal of the E1a enhancer elements (-141 to –305 relative to the E1a cap site at +1) and the insertion of a poly(A) signal sequence downstream of the left end ITR are sufficient for efficient transcription termination. An adenovirus backbone (Ar6F), with deletion from nucleotides 104 to 551, and another

adenovirus backbone version, Ar6pAF, that combines the E1a deletion and the SV40 early poly(A) signal insertion upstream of the E1a gene are generated (see Example 1). In both vectors the packaging sequences are moved upstream of the right ITR. To measure read-through upstream of the E1a gene, an E1a FACS-assay is used that quantifies the levels of E1 protein (see Example 2). Of the two adenoviral backbones generated, the Ar6pAF shows a reduction of E1a expression of approximately 96%. These results show that it is possible to selectively control E1a gene activity by placing the gene under the control of a tissue specific promoter, if an insulating sequence is placed immediately upstream of the promoter.

Thus, in a preferred embodiment, the adenoviral vector comprises a deletion 5' to the termination signal sequence. A deletion in the packaging signal 5' to the termination signal sequence may be such that the packaging signal becomes non-functional. In one specific embodiment, the deletion comprises a deletion 5' to the termination signal sequence wherein the deletion spans at least the nucleotides 189 to 551. In another preferred embodiment, the deletion comprises a deletion 5' to the termination signal sequence wherein the deletion spans at least nucleotides 103 to 551. In these situations, it is preferred that the packaging signal is located (i.e. re-inserted) at a position 3' to the termination signal sequence.

The viral vectors of the invention may be "replication-conditional vectors". Replication-conditional vectors are vectors which when introduced into a tissue will not replicate, or will only replicate to a minimal extent, unless a transcriptional regulatory sequence in that vector is activated or derepressed in that tissue. For example, a gene that is essential for replication may be modified by replacing the transcriptional regulatory sequence on which transcription of that gene normally depends with a heterologous transcriptional regulatory sequence. This transcriptional regulatory sequence depends upon the presence of transcriptional regulatory factors or the absence of transcriptional regulatory inhibitors. The presence of these factors in a given tissue or the absence of such inhibitors in a given tissue provides the replication-conditionality. Alternatively, the native transcriptional regulatory sequence may be disabled or rendered dysfunctional by partial removal or other mutation (one or more base changes, insertions, inversions, etc.). Replication-conditional vectors and methods for obtaining such viral vectors are further described in US patent 5,998,205 (Hallenbeck et al.) which is hereby incorporated by reference in its entirety.

The term "replication" is used according to its art-recognized meaning. The essential feature is that nucleic acid copies of the original viral vector are synthesized. In the case of DNA viruses, replication at the nucleic acid level is DNA replication. In the case of RNA viruses, nucleic acid replication is replication into plus or minus strand (or both). In the case of retroviruses, replication at the nucleic acid level includes the production of cDNA as well as the further production of RNA viral genomes. Replication also includes the formation of infectious DNA or RNA viral particles. Such particles may successively infect cells in a given target tissue, thus distributing the vector through all or a significant portion of the target tissue.

In a preferred embodiment of the invention, the transcription unit to be shielded from the interfering genetic element comprises a gene essential for replication. For example, if the vector construct of the invention is an adenoviral vector, the gene essential for replication may be selected from the group consisting of the E1a, E1b, E2 and E4 coding sequences and most preferredly the gene essential for replication is selected from the group consisting of the E1a coding sequence and the E1b coding sequence. Particularly preferred is the adenoviral E1a gene as the gene essential for replication. The term "gene essential for replication" refers to a genetic sequence whose transcription is required for the vector to replicate in the target cell.

In a further embodiment of the invention, a tissue-specific transcritional regulatory sequence is operably linked to said gene essential for replication.

The term "tissue-specific" is intended to mean that the transcriptional regulatory sequence to which the gene essential for replication is operably linked functions specifically in that tissue so that replication proceeds in that tissue. This can occur by the presence in that tissue, and not in non-target tissues, of transcription factors that activate the transcriptional regulatory sequence. It can also occur by the absence of transcription inhibiting factors that normally occur in non-target tissues and prevent transcription as a result of the transcription regulatory sequence. Tissue specificity is particularly relevant in the treatment of the abnormal counterpart of a normal tissue. Such counterparts include, but are not limited to, liver tissue and liver cancer, lung tissue and lung cancer, breast tissue and breast cancer, colon tissue and colon cancer, prostate tissue and prostate cancer, and melanoma and normal skin tissue. Tissue specificity also includes the presence of an abnormal tissue type

interspersed with normal tissue of a different tissue type, as for example in the case of metastases of colon cancer, breast cancer, lung cancer, prostate cancer, and the like, into tissue such as liver. In this case, the target tissue is the abnormal tissue, and tissue specificity reflects the restriction of vector replication to the abnormal tissue interspersed in the normal tissue. Tissue specificity, in the context of treatment, is particularly relevant in vivo. However, ex vivo treatment and tissue replacement also falls within the concept of tissue specificity according to the present invention.

The term "transcriptional regulatory sequence" is intended to mean any DNA sequence which can cause the linked gene to be either up- or down-regulated in a particular cell, such as for example promoter and enhancers. Various combinations of transcriptional regulatory sequences can be included in a vector. One or more may be heterologous. Further, one or more may have tissue-specificity. For example, a single transcriptional regulatory sequence could be used to drive replication by more than one gene essential for replication. This is the case, for example, when the gene product of one of the genes drives transcription of the further gene(s). An example for the case of an adenoviral vector is a heterologous promoter linked to a cassette containing an E1a coding sequence (E1a promoter deleted) and the entire E1b gene. In such a cascade, only one heterologous transcriptional regulatory sequence may be necessary. When genes are individually (separately) controlled, however, more than one transcriptional regulatory sequence can be used if more than one such gene is desired to control replication.

In a preferred embodiment the tissue-specific transcriptional regulatory sequence is a promoter or an enhancer. Preferredly, the promoter is selected from the group consisting of an E2F-responsive promoter, preferredly E2F-1, CEA, MUC1/DF3, alpha-fetoprotein, erb-B2, surfactant, tyrosinase, PSA, TK, p21, hTERT, hKLK2, probasin and cyclin gene derived promoters. The enhancer preferredly is selected from the group consisting of DF3, breast cancer-specific enhancer, PSA, viral enhancers, and steroid receptor enhancers.

The adenoviral vectors of the invention may in particular be oncolytic adenoviral vectors. Oncolytic adenoviral vectors are adenoviral vectors which selectively replicate in tumor cells and destroy the cells in which they replicate, but do not replicate to any significant degree, in non-tumor cells. For example, oncolytic adenoviral vector may have a tissue-specific transcritional regulatory sequence is operably linked to said gene essential for replication as

described above. Alternatively, oncolytic adenoviral particles may include a mutation in a gene essential for adenoviral replication, such as the E1a or E1b genes. Such mutations may render adenoviral replication specific for tumor tissue, e.g. if the cells of said tissue have a defect in the p53 or Rb pathways. Oncolytic adenoviral vectors may or may not include a heterologous gene in addition to the adenoviral elements necessary for replication.

The present invention provides an oncolytic adenoviral vector, Ar6pAE2fF, that utilizes the E2F-1 promoter to drive expression of the E1a gene. The E2F-1 promoter is selectively activated in Rb pathway defective tumor cells. Transduction of A549 cells with the Ar6pAE2fF vector results in expression of E1a, indicating that this expression is dependent on the activity of the E2F-1 promoter. This result is consistent with the fact that A549 cells are defective in p16, a member of the Rb pathway. The activity of the E2F-1 promoter in Ar6pAE2fF has been also confirmed in several tumor cell lines.

In the field of cancer therapy with oncolytic adenoviral vectors, the present invention may increase the therapeutic effect because the use of an insulating sequence will reduce replication and toxicity of the oncolytic adenoviral vectors in non-target cells. Oncolytic vectors of the present invention with a polyadenylation signal inserted upstream of E1a coding region are superior to their non-modified counterparts as they demonstrated the lowest level of E1a expression (see Example 2). Thus, insertion of a polyadenylation signal sequence to stop nonspecific transcription from the left ITR will improve the specificity of E1a expression from the respective promoter. Insertion of the polyadenylation signal sequences will reduce replication of the oncolytic adenoviral vector in nontarget cells and therefore toxicity.

The present invention, in one aspect, also provides a method of reducing the transcription level of a transcription unit in a vector construct caused by an interfering genetic element that displays enhancer or promoter activity in relation to said transcription unit, comprising the steps of identifying a suitable insulating sequence and inserting said insulating sequence into said vector construct 5' to said transcription unit. In a preferred embodiment, the transcrition level is reduced at least about 10-fold, preferredly at least about 20, 50 or 200-fold as compared to an equivalent dose of viral vectors not including the insulating sequence.

In a further embodiment, the present invention provides vector constructs which include a therapeutic gene. A therapeutic gene can be one that exerts its effect at the level of RNA or protein. For instance, a protein encoded by a therapeutic gene can be employed in the treatment of an inherited disease, e.g., the use of a cDNA encoding the cystic fibrosis transmembrane conductance regulator in the treatment of cystic fibrosis. Further, the protein encoded by the therapeutic gene can exert its therapeutic effect by causing cell death. For instance, expression of the protein, itself, can lead to cell death, as with expression of diphtheria toxin A, or the expression of the protein can render cells selectively sensitive to certain drugs, e.g., expression of the Herpes simplex (HSV) thymidine kinase gene renders cells sensitive to antiviral compounds, such as acyclovir, gancyclovir and FIAU (1-(2-deoxy-2-fluoro-.beta.-D-arabinofuranosil)-5-iodouracil). Alternatively, the therapeutic gene can exert its effect at the level of RNA, for instance, by encoding an antisense message or ribozyme, a protein that affects splicing or 3' processing (e.g., polyadenylation), or a protein that affects the level of expression of another gene within the cell, e.g. by mediating an altered rate of mRNA accumulation, an alteration of mRNA transport, and/or a change in post-transcriptional regulation.

DNA sequences encoding therapeutic genes which may be placed into the vector construct include, but are not limited to, DNA sequences encoding tumor necrosis factor (TNF) genes, such as TNF-α; genes encoding interferons such as interferon-α, interferon-ß, and interferon-gamma; genes encoding interleukins such as IL-1, IL-1ß, and Interleukins 2 through 14; genes encoding GM-CSF; genes encoding adenosine deaminase, or ADA; genes which encode cellular growth factors, such as lymphokines, which are growth factors for lymphocytes; genes encoding soluble CD4; Factor VIII; Factor IX; T-cell receptors; the LDL receptor, ApoE, ApoC,ApoAI and other genes involved in cholesterol transport and metabolism; the alpha-1 antitrypsin gene, the ornithine transcarbamylase gene, the CFTR gene, the insulin gene, negative selective markers or "suicide" genes, such as viral thymidine kinase genes, such as the Herpes Simplex Virus thymidine kinase gene, the cytomegalovirus virus thymidine kinase gene, and the varicella-zoster virus thymidine kinase gene; Fc receptors for antigen-binding domains of antibodies, and antisense sequences which inhibit viral replication. The DNA sequence encoding the therapeutic gene may preferredly be selected from either GM-CSF, thymidine kinase, Nos, FasL, or sFasR (soluble Fas receptor).

The DNA sequence encoding the therapeutic agent may also be a sequence which is a part of the adenoviral genome, such as the adenoviral E1a gene. On one hand, E1a is instrumental in driving the adenoviral replication cycle, which in turn leads to cell lysis. Accordingly, E1a may be considered a DNA sequence encoding the therapeutic agent within the meaning of the invention if administered to, for example, a tumor tissue. Furthermore, such genes may provide an additional therapeutical benefit, e.g. by sensitizing the infected cell to certain agents and/or radiation.

For human patients, the therapeutic gene will generally be of human origin although genes of closely related species that exhibit high homology and biologically identical or equivalent function in humans may be used if the gene does not produce an adverse immune reaction in the recipient. A therapeutically effective amount of a nucleic acid sequence or a therapeutic gene is an amount effective at dosages and for a period of time necessary to achieve the desired result. This amount may vary according to various factors, including but not limited to sex, age, weight of a subject, and the like.

The DNA sequence encoding at least one therapeutic gene is under the control of a suitable promoter. Suitable promoters which may be employed include, but are not limited to, adenoviral promoters, such as the adenoviral major late promoter; or hetorologous promoters, such as the cytomegalovirus (CMV) promoter; the Rous Sarcoma Virus (RSV) promoter; inducible promoters, such as the MMT promoter, the metallothionein promoter; heat shock promoters; the albumin promoter; and the ApoAI promoter. In a preferred embodiment, the promoter of the invention is an E2F-responsive promoter, in particular the E2F-1 promoter. In one embodiment of this invention, the E2F promoter is operatively linked to the E1a gene.

In addition to the E2F promoter, the following tumor selective promoters are preferredly contemplated in this invention: osteocalcin, L-plastin, CEA, AVP, c-myc, telomerase, skp-2, psma, cyclin A, and cdc25 promoters. It is to be understood, however, that the scope of the present invention is not to be limited to specific foreign genes or promoters. The selection of a particular promoter and enhancer depends on what cell type is to be used to express the protein of interest. Some eukaryotic promoters and enhancers have a broad host range while others are functional in a limited subset of cell types.

The viral vectors of the invention are useful for the delivery of genes to eukaryotic cells, for example, in order express the delivered genes and study their respective functions. Preferably, the cell is a mammalian cell. More preferably, the mammalian cell is a primate cell. Most preferably, the primate cell is a human cell. The viral vectors are also useful in studying cell transduction and gene expression in animal models.

The viral vectors are also useful for gene therapy. In particular, the expression of genes delivered by the viral vectors of the invention is useful to modify the properties of transfected cells in a pre-determined fashion for purposes of prophylaxis or therapy of disease.

Accordingly, in a further aspect, the present invention also provides a eukaryotic cell transfected with the vector construct of the invention. Preferably, the cell is a mammalian cell. More preferably, the mammalian cell is a primate cell. Most preferably, the primate cell is a human cell.

The term "transfection" as used herein refers to the introduction of foreign DNA into eukaryotic cells. Transfection may be accomplished by a variety of means known to the art, including calcium phosphate-DNA co-precipitation, DEAE-dextran-mediated transfection, polybrene-mediated transfection, electroporation, microinjection, liposome fusion, lipofection, protoplast fusion, biolistics and viral infection.

Cells which may be transfected / infected by the vector constructs of the invention include, but are not limited to, primary cells, such as primary nucleated blood cells, such as leukocytes, granulocytes, monocytes, macrophages, lymphocytes (including T-lymphocytes and B-lymphocytes), totipotent stem cells, and tumor infiltrating lymphocytes (TIL cells); bone marrow cells; endothelial cells; including activated endothelial cells; epithelial cells; keratinocytes; stem cells; hepatocytes, including hepatocyte precursor cells; fibroblasts; mesenchymal cells; mesothelial cells; parenchymal cells; vascular smooth muscle cells; brain cells and other neural cells; gut enterocytes; gut stem cells; and myoblasts. Cells which may be infected further include primary and metastatic cancer cells, including, but not limited to prostate, breast, pancreatic, lung, including both small cell and non-small cell lung cancers, colon, and liver cancers.

A cell can be present as a single entity, or can be part of a larger collection of cells. Such a "larger collection of cells" can comprise, for instance, a cell culture (either mixed or pure), a tissue, e.g., epithelial or other tissue such as a neoplastic (benign or malign) tissue, an organ (e.g., heart, lung, liver and other organs), an organ system (e.g., circulatory system, respiratory system, gastrointestinal system, or other organ system), or an organism (e.g., a bird, mammal, or the like). In one embodiment , the cells being targeted are of the circulatory system (e.g., including, but not limited to heart, blood vessels, and blood), respiratory system (e.g., nose, pharynx, larynx, trachea, bronchi, bronchioles, lungs, and the like) or the gastrointestinal system (e.g., including mouth, pharynx, esophagus, stomach, intestines, salivary glands, pancreas, liver, gallbladder, and others). In a preferred embodiment cells of neoplastic tissue (i.e. 'tumor tissue') are targeted with the targeting molecule/adenoviral particle complex of the invention.

EXAMPLES

The invention will now be described with respect to the following examples; it is to be understood, however, that the scope of the present invention is not intended to be limited thereby.

Example 1: Construction and molecular characterization of replication-selective adenoviruses Ar6F, Ar6pAF and Ar6pAE2fF

Two adenovirus backbones that were expected to minimize nonspecific activation of the E1a gene were developed. The Ar6F adenoviral vector contains the left side ITR directly linked to the E1a coding region, with the intervening nucleotides deleted (nucleotides 104-551 in the Ad5 sequence, GenBank accession number M73260) and replaced with a multiple cloning site (Fig 4). The Ar6pAF adenoviral vector is identical to Ar6F except that it contains the 145 nucleotide SV-40 early poly(A) signal inserted between the left ITR and the E1a coding region (Fig 5). In both of these vectors, the packaging signal normally present near the left ITR was moved to the right ITR (Fig 3, panel B). This was performed by replacing the right ITR with the reverse complementary sequence of the first 392bp of Ad5, which contains the left ITR and the packaging signal. Finally, to generate the adenoviral vector Ar6pAE2fF, the

tumor selective promoter E2F-1 was inserted between the SV-40 early poly(A) signal and the E1a coding region present in Ar6pAF (Fig 3, panel A).

The first 1802 nucleotides of the Ar6pAE2fF adenoviral vector, including the ITR, poly(A), E2F-1 promoter and the E1a gene was confirmed by DNA sequencing. In addition, the last 531 nucleotides at the right end of the vector, containing the packaging signal and right ITR was confirmed by sequencing (Fig 3).

Adenoviral genomes containing these modifications were cloned by standard methods in bacterial plasmids. Homologous recombination in E. coli was performed between these bacterial shuttle plasmids containing fragments of the Ad genome to generate plasmids containing full-length infectious viral genomes (He et al., 1998. A simplified system for generating recombinant adenoviruses. PNAS 95, 2509-2514). These plasmids containing full length adenoviral genomes were linearized with a restriction enzyme to release the adenoviral genome DNA from the bacterial plasmid sequences. The adenoviral DNA was then transfected into a complementing cell line AE1-2a (Gorziglia et al., 1996. Elimination of both E1 and E2a from adenovirus vectors further improves prospects for in vivo human gene therapy. J. Virol 6,4173-4178) using the LipofectaAMINE-PLUS reagent system (Life Technologies, Rockville, MD). The cells were incubated at 37°C for approximately 5-7 days. Adenovirus was amplified and purified by CsCl gradient as described (Jakubczak et al., 2001 Adenovirus type 5 viral particles pseudotyped with mutagenized fiber proteins show diminished infectivity of coxsackie B-Adenovirus receptor-bearing cells. J. Virol. 75:2972-2981). Virus particle concentrations were determined by spectrophotometric analysis (Mittereder et al., 1996. Evaluation of the concentration and bioactivity of adenovirus vectors for gene therapy. J Virol 70, 7498-7509).

1.2 Viral DNA isolation and Southern analysis

DNA was isolated from CsCl-purified virus preparation as described (Puregene Kit, Gentra). Viral DNA was digested with the indicated restriction enzymes and analyzed on 1% agarose/TAE gels containing ethidium bromide. A total of 1 ug of each DNA sample was digested with ClaI, XbaI, HpaI, SalI and BamHI and subjected to Southern analysis according to standard procedures. The probe was prepared by random oligonucleotide priming and contained the E2F-1 promoter.

Figure 6 summarizes the cloning and structures of Ar6pAF and Ar6pAE2fF vectors. The DNA structure of a research lot of Ar6pAE2fF vector was confirmed by Southern analysis. The expected left DNA region fragments were obtained using five independent restriction endonucleases. Southern blot analysis with an E2F promoter DNA probe demonstrated the expected hybridization pattern for all restriction endonucleases. Thus, these results confirmed the presence of the E2F-1 promoter in the correct position and verified the integrity of the viral DNA.

1.3 Limiting Dilution Cloning of Ar6pAE2fF vector in PER.C6 cells

A seed lot of Ar6pAE2fF vector was produced for further evaluations. To obtain a pure seed lot of a virus it is necessary to isolate a clone derived from a single virus particle. The cloning of Ar6pAE2fF virus was accomplished through viral limiting dilution as described in below.

Ten 96 well plates of PER.C6 cells (Fallaux et al., 1998. New helper cells and matched early region 1-deleted adenovirus vectors prevent generation of replication-competent adenoviruses. Human. Gene Ther 9, 1909-1917) were plated at 5 x 103 cells/well in 0.04 ml volume /well. PER.C6 cells were grown in DMEM with the addition of 10% FBS and 10 mM MgCl2. 10 ul of Ar6pAE2fF containing 1 x 10-2 particles/ul was added to each well, giving a final infection of 0.1 particle/well. Infected cells were incubated at 37 °C and 5% CO2 for 4 hours, after which 150 ul of media was added. The virus infected cells were incubated at 37 °C and 5% CO2 for 12 days followed by scoring for CPE. The 0.1 particle /cells clones 7-9 from PER.C6 cells were harvested on day 13.Three clones, 7-9 showed CPE and were freeze thawed 5 times and amplified on PER.C6 cells plated in 6 well dishes. On day 3, CVL were prepared from clones 7-9 and clone 7 was further amplified in a T150 of PER.C6 cells. Ar6pAE2fF clone 7 T150 was harvested 2 days post-infection, a time at which the cells had reached complete CPE. The CVL was freeze thawed 5 times and cellular debris was spun out. A T75 flask of PER.C6 cells was plated and infected with 0.5 ml of the above CVL.

Of the 960 wells infected with 0.1 particle/cell, three wells showed CPE. These 3 clones were in the range of the theoretical numbers of clones expected. Statistically, only 4 wells out of the 10 plates should give CPE. This gives odds of 1:2500 that there will be more than

one infectious particle/well when assuming a particle:pfu ratio of 25. The three clones were amplified in PER.C6 cells and the genome of clone 7 showed the expected size DNA fragments when analyzed with HpaI, XhoI and XbaI restriction endonuclease.

1.4 Sequence analysis.

The 5'-end first 1802 nucleotides and the last 3'-end nucleotides from bp 33881-34412 of the plasmids pDL6pAE2f and Ar6pAE2fF clone 7 were directly sequenced.

Regions in Accessionary Seed lot confirmed by DNA Sequencing

```
  1   CATCATCAATAATATACCTTATTTTGGATTGAAGCCAATATGATAATGAGGGGGTGGAGT
      +-------------------------ITR-------------------------------

 61   TTGTGACGTGGCGCGGGGCGTGGGAACGGGGCGGGTGACGTAGGGCGCGATCAAGCTTAT
      +-------------------------ITR------------+           +----

121   CGATACCGTCGAAACTTGTTTATTGCAGCTTATAATGGTTACAAATAAAGCAATAGCATC
      ---------------------------polyA-----------------------------

181   ACAAATTTCACAAATAAAGCATTTTTTTCACTGCATTCTAGTTGTGGTTTGTCCAAACTC
      ---------------------------polyA-----------------------------

241   ATCAATGTATCTTATCATGTCTGGATCCGGCGCCGCTAGCGATCATCCGGACAAAGCCTGC
      --------------------+                     +-------------------

301   GCGCGCCCCGCCCCGCCATTGGCCGTACCGCCCCGCGCCGCCGCCCCATCTCGCCCCTCG
      ------------------------E2F-1 promoter----------------------

361   CCGCCGGGTCCGGCGCGTTAAAGCCAATAGGAACCGCCGCCGTTGTTCCCGTCACGGCCG
      ------------------------E2F-1 promoter----------------------

421   GGGCAGCCAATTGTGGCGGCGCTCGGCGGCTCGTGGCTCTTTCGCGGCAAAAAGGATTTG
      ------------------------E2f-1 promoter----------------------

481   GCGCGTAAAAGTGGCCGGGACTTTGCAGGCAGCGGCGGCCGGGGGCGGAGCGGGATCGAG
      ------------------------E2f-1 promoter----------------------

541   CCCTCGATGATATCAGATCATCGGATCCCGGTCGACTGAAAATGAGACATATTATCTGCC
      --------------+                  +--------------------------

601   ACGGAGGTGTTATTACCGAAGAAATGGCCGCCAGTCTTTTGGACCAGCTGATCGAAGAGG
      ------------------------Ela gene----------------------------

661   TACTGGCTGATAATCTTCCACCTCCTAGCCATTTTGAACCACCTACCCTTCACGAACTGT
      ------------------------Ela gene----------------------------
```

721 ATGATTTAGACGTGACGGCCCCCGAAGATCCCAACGAGGAGGCGGTTTCGCAGATTTTTC
----------E1a gene----------

781 CCGACTCTGTAATGTTGGCGGTGCAGGAAGGGATTGACTTACTCACTTTTCCGCCGGCGC
----------E1a gene----------

841 CCGGTTCTCCGGAGCCGCCTCACCTTTCCCGGCAGCCCGAGCAGCCGGAGCAGAGAGCCT
----------E1a gene----------

901 TGGGTCCGGTTTCTATGCCAAACCTTGTACCGGAGGTGATCGATCTTACCTGCCACGAGG
----------E1a gene----------

961 CTGGCTTTCCACCCAGTGACGACGAGGATGAAGAGGGTGAGGAGTTTGTGTTAGATTATG
----------E1a gene----------

1021 TGGAGCACCCCGGGCACGGTTGCAGGTCTTGTCATTATCACCGGAGGAATACGGGGGACC
----------E1a gene----------

1081 CAGATATTATGTGTTCGCTTTGCTATATGAGGACCTGTGGCATGTTTGTCTACAGTAAGT
----------E1a gene----------

1141 GAAAATTATGGGCAGTGGGTGATAGAGTGGTGGGTTTGGTGTGGTAATTTTTTTTTTAAT
----------E1a gene----------

1201 TTTTACAGTTTTGTGGTTTAAAGAATTTTGTATTGTGATTTTTTTAAAAGGTCCTGTGTC
----------E1a gene----------

1261 TGAACCTGAGCCTGAGCCCGAGCCAGAACCGGAGCCTGCAAGACCTACCCGCCGTCCTAA
----------E1a gene----------

1321 AATGGCGCCTGCTATCCTGAGACGCCCGACATCACCTGTGTCTAGAGAATGCAATAGTAG
----------E1a gene----------

1381 TACGGATAGCTGTGACTCCGGTCCTTCTAACACACCTCCTGAGATACACCCGGTGGTCCC
----------E1a gene----------

1441 GCTGTGCCCCATTAAACCAGTTGCCGTGAGAGTTGGTGGGCGTCGCCAGGCTGTGGAATG
----------E1a gene----------

1501 TATCGAGGACTTGCTTAACGAGCCTGGGCAACCTTTGGACTTGAGCTGTAAACGCCCCAG
----------E1a gene----------

1561 GCCATAAGGTGTAAACCTGTGATTGCGTGTGTGGTTAACGCCTTTGTTTGCTGAATGAGT
----------E1a gene----------

1621 TGATGTAAGTTTAATAAAGGGTGAGATAATGTTTAACTTGCATGGCGTGTTAAATGGGGC
----------+----------

1681 GGGGCTTAAAGGGTATATAATGCGCCGTGGGCTAATCTTGGTTACATCTGACCTCATGGA
----------E1b gene----------

1741 GGCTTGGGAGTGTTTGGAAGATTTTTCTGCTGTGCGTAACTTGCTGGAACAGAGCTCTAA

```
      -------------------------E1b gene---------------------------

1801  CA
      --


33881  AACCTACGCCCAGAAACGAAAGCCAAAAAACCCACAACTTCCTCAAATCGTCACTTCCGT


33941 TTTCCCACGTTACGTCACTTCCCATTTTAATTAAGAATTCTACAATTCCCAACACATACA


34001 AGTTACTCCGCCCTAAAACCCTGGGCGAGTCTCCACGTAAACGGTCAAAGTCCCCGCGGC
                        +-packaging signal----------------------

34061 CCTAGACAAATATTACGCGCTATGAGTAACACAAAATTATTCAGATTTCACTTCCTCTTA
      --------------------packaging signal----------------------

34121 TTCAGTTTTCCCGCGAAAATGGCCAAATCTTACTCGGTTACGCCCAAATTTACTACAACA
      --------------------packaging signal----------------------

34181 TCCGCCTAAAACCGCGCGAAAATTGTCACTTCCTGTGTACACCGGCGCACACCAAAAACG
      -----------------------------------+

34241 TCACTTTTGCCACATCCGTCGCTTACATGTGTTCCGCCACACTTGCAACATCACACTTCC


34301 GCCACACTACTACGTCACCCGCCCCGTTCCCACGCCCCGCGCCACGTCACAAACTCCACC
            +-----------------ITR-----------------------------

34361 CCCTCATTATCATATTGGCTTCAATCCAAAATAAGGTATATTATTGATGATG
      ---------------------------ITR--------------------+
```

Regions of Ar6pAE2fF from seedlot #TCA 254 confirmed by DNA sequencing. Regions in first 1802 nucleotides are ITR (nucleotides 1-103), poly-adenylation signal (nucleotides 116-261), human E2F-1 promoter (nucleotides 283-555), E1a gene (nucleotides 574-1647) and a portion of the E1b gene (nucleotides 1648-1802) are indicated. Regions in the last 531 nucleotides are the PacI restriction site (nucleotides 33967-33974) (underlined), the packaging signal (nucleotides 34020-34217 and the ITR (34310-34412).

Example 2: Characterization of E1a expression by FACS

To determine if deletions of enhancer elements and insertion of a polyA signal may be sufficient for efficient transcription termination, a quantitative E1a FACS assay was used to evaluate E1a expression in a non-complementing A549 cell background (p16- p53+ Rb+).

We compared the E1a expression from cells infected with Addl327, Addl312, Ar6F, Ar6pAF or Ar6pAE2fF at doses of 10, 50, 250 and 1250 virus particles per cell (VPC) (Table 1). The highest level of E1a expression was observed with the Addl327 at all range of doses. In contrast, as expected the E1a deleted mutant Addl312 showed no E1a expression. Under the conditions used in this experiment (10 to 1250 VPC) there was about 80% to 22% less E1a detected in cells transduced with Ar6F than in those transduced with Addl327. The E1a expression in cells transduced with Ar6pAF was significantly reduced about 100% to 96%, in all doses, as compared to the expression from cells infected with the Addl327. The expression of E1a from cells infected with the Ar6pAE2fF oncolytic vector was reduced 50% as compared with the Addl327 virus at a dose of 50VPC.

In conclusion, the insertion of a poly(A) signal in the Ar6pAF vector reduced the E1a expression in A549 cells. In contrast, insertion of the E2F-1 promoter reestablished the E1a expression, thus demonstrating that E1a expression was exclusively due to the inserted promoter.

	10 vpc	50 vpc	250 vpc	1250 vpc
Addl327	27.5 $\pm$2.2	72.9 $\pm$3.8	94.4 $\pm$0.7	98.4 $\pm$0.4
Addl312	0.0 $\pm$0.0	0.0 $\pm$0.0	0.0 $\pm$0.0	0.0 $\pm$0.0
Ar6F	5.6 $\pm$0.8	28.3 $\pm$1.1	59.4 $\pm$4.7	76.9 $\pm$3.6
Ar6pAF	0.0 $\pm$0.0	0.1 $\pm$0.1	0.3 $\pm$0.1	3.8 $\pm$2.4
Ar6pAE2fF	ND	39.7 $\pm$0.1	ND	ND

Table 1. E1a expression in A549 noncomplementing cells.

Noncomplementing A549 cells were infected with either vector at 10, 50, 250 and 1250 VPC. E1a expression was determined 24 hours postinfection by FACS.

Protocol for E1a FACS Assays

Cells were plated the day before infection in 12-well plates. The next day, media was aspirated from cells, virus dose formulations in particles per cell were added to the wells and the plates were rocked at 37°C for 4 hours. Virus/media was aspirated, washed one time, then replaced with complete growth media and incubated 20 hours at 37°C. Cells were harvested by trypsin-EDTA digestion, and fixed in 70% ethanol for 20 minutes at room temperature. Then the cells were washed one time and resuspended in FACS buffer (PBS, 3% FBS, 0.1% NaN_3). 10µl of a 1:10 dilution of unconjugated anti-E1a antibody (Calbiochem, Anti-Adenovirus 2E1A, Human (Ab-1)) or mouse IgG_{2a} isotype control (Sigma M-5409) was added and incubated at room temperature for 30 minutes. The cells were washed one time with FACS buffer. Then 50µl of 1:40 dilution of GAM PE (Sigma P-9670) was added and incubated at room temperature for 30 minutes. Then the cells were washed, resuspended in 200µl FACS buffer, and 20,000 events on FACSCAN were acquired.

Example 3: Toxicity of adenoviral vectors

Acute hepatic toxicity in Balb/c SCID male mice is used to distinguish between adenoviral vectors with different levels of E1a activity. A profound difference in serum liver enzyme elevations is observed between vectors with wild-type E1a expression and those with minimal or silent E1a expression.

Studies were designed with ten animals per group. Control groups were HBSS vehicle alone, the negative control E1a-deleted Addl312 and the E1a-containing positive control Addl327. Viruses were injected at a dose of 6.25×10^{11} particles/kg intravenously into the tail vein in a volume of 10ml/kg; an equivalent dose volume of HBSS (10 mL/kg) was injected in the vehicle control group. Animals were injected on study day 1, with an interim sacrifice of half of each group on study day 4 and a terminal sacrifice of the remaining animals on study day

15. On study days 4 and 15, serum was collected from all mice, and the livers removed from the animals scheduled for sacrifice (5/group). In addition, body weights were measured on all surviving mice on study days –3, 1, 3, 4, 8 and 15.

The acute toxicity of E1a-containing adenoviral vectors in the backbones Ar6F, Ar6PAF was compared. Viruses are prepared as described in Example 1. Based on body weight change (Figure 7, map of constructs see Figure 8) and serum ALT and AST levels (Table 2), the hepatotoxicity of Ar6F was higher than Ar6pAF.

Table 2:

	ALT		AST		DB	
Vector	mean	sd	mean	sd	mean	sd
Ar6F	2213.40	1018.61	1500.40	922.53	0.19	0.33
Ar6pAF	57.6*	24.59	130.7*	40.33	0.01*	0.03

*significant difference versus Ar6F ($p<0.05$)

The disclosures of all patents, publications (including published patent applications), and database accession numbers referred to in this specification are specifically incorporated herein by reference in their entirety to the same extent as if each such individual patent, publication and database number were specifically and individually indicated to be incorporated by reference in its entirety.

WHAT IS CLAIMED IS:

1. A viral vector having at least one interfering genetic element and comprising at least one transcription unit, wherein at least one insulating sequence is located 5' to the transcription initiation site of said transcription unit and 3' to said interfering genetic element.

2. The viral vector of claim 1 wherein said insulating sequence is located no more than 3000 nucleotides 5' to the transcription initiation site of said transcription unit.

3. The viral vector of claim 1 wherein said transcription unit is the first transcription unit from the 5' end of said viral vector.

4. The viral vector of claim 1 wherein said insulating sequence is a termination signal sequence.

5. The viral vector of claim 4 wherein the termination signal sequence is a polyadenylation signal sequence.

6. The viral vector of claim 5 wherein the polyadenylation signal sequence is the SV40 late polyadenylation signal sequence.

7. The viral vector of claim 5 wherein the polyadenylation signal sequence is the SV40 early polyadenylation signal sequence.

8. The viral vector of claim 1 further comprising a therapeutic gene.

9. A viral vector particle comprising the viral vector of claim 1.

10. A eukaryotic cell transfected with the viral vector particle of claim 9.

11. The vector of claim 1 which is an adenoviral vector.

12. The adenoviral vector of claim 11 wherein the vector construct comprises an adenoviral 5'ITR, an adenoviral 3'ITR and an adenoviral packaging signal.

13. The adenoviral vector of claim 11 wherein the interfering genetic element is located within the 5'ITR.

14. The adenoviral vector of claim 11 wherein the interfering genetic element is located between –141 and -305 relative to the E1a transcription initiation site at +1.

15. The adenoviral vector of claim 11 further comprising a deletion 5' to the termination signal sequence.

16. The adenoviral vector of claim 15 comprising a deletion in the packaging signal 5' to the termination signal sequence such that the packaging signal becomes non-functional.

17. The adenoviral vector of claim 15 comprising a deletion 5' to the termination signal sequence wherein the deletion spans at least nucleotides 189 to 551.

18. The adenoviral vector of claim 17 comprising a deletion 5' to the termination signal sequence wherein the deletion spans at least nucleotides 103 to 551.

19. The adenoviral vector of claim 11 wherein the packaging signal is located 3' to the termination signal sequence.

20. The adenoviral vector of claim 11 wherein the transcription unit comprises a gene essential for replication.

21. The adenoviral vector of claim 20 wherein the gene essential for replication is selected from the group consisting of E1a, E1b, E2 and E4 coding sequences.

22. The adenoviral vector of claim 21 wherein the gene essential for replication is selected from the group consisting of E1a and E1b coding sequences.

23. The adenoviral vector of claim 20 wherein a tissue-specific transcritional regulatory sequence is operably linked to said gene essential for replication.

24. The adenoviral vector of claim 23 wherein said tissue-specific transcriptional regulatory sequence is a promoter or an enhancer.

25. The adenoviral vector of claim 24 wherein said promoter is selected from the group consisting of E2F, CEA, MUC1/DF3, alpha-fetoprotein, erb-B2, surfactant, tyrosinase, PSA, TK, p21, hTERT, hKLK2, probasin and cyclin gene derived promoters.

26. The adenoviral vector of claim 24 wherein said enhancer is selected from the group consisting of DF3, breast cancer-specific enhancer, viral enhancers, and steroid receptor enhancers.

27. The adenoviral vector of claim 11 further comprising a deletion in the E3 region.

28. The adenoviral vector of claim 11 further comprising a therapeutic gene.

29. An adenoviral vector particle comprising the adenoviral vector of claim 11.

30. A eukaryotic cell transfected with the adenoviral vector particle of claim 29.

31. A method of reducing the transcription level of a transcription unit in a viral vector caused by an interfering genetic element which displays enhancer or promoter activity in relation to said transcription unit, comprising the steps of identifying a suitable insulating sequence and inserting said insulating sequence into said viral vector 5' to the transcription initiation site of said transcription unit.

32. The method of claim 31 wherein said insulating sequence is located no more than 3000 nucleotides 5' to the transcription initiation site of said transcription unit.

33. The method of claim 31 wherein said insulating sequence is a termination signal sequence.

34. The method of claim 33 wherein the termination signal sequence is a polyadenylation signal sequence.

35. The method of claim 34 wherein the polyadenylation signal sequence is the SV40 late polyadenylation signal sequence.

36. The method of claim 34 wherein the polyadenylation signal sequence is the SV40 early polyadenylation signal sequence.

37. The method of claim 31 wherein the vector construct further comprises a therapeutic gene.

38. The adenoviral vector of claims 20, 21, or 22 further comprising a therapeutic gene.

39. The adenoviral vector of claim 38, wherein said therapeutic gene is a cytokine.

40. The adenoviral vector of claim 39, wherein said cytokine is GM-CSF.

Fig. 1 Cleavage and Polyadenylation Process For The SV40 early Poly(A) site

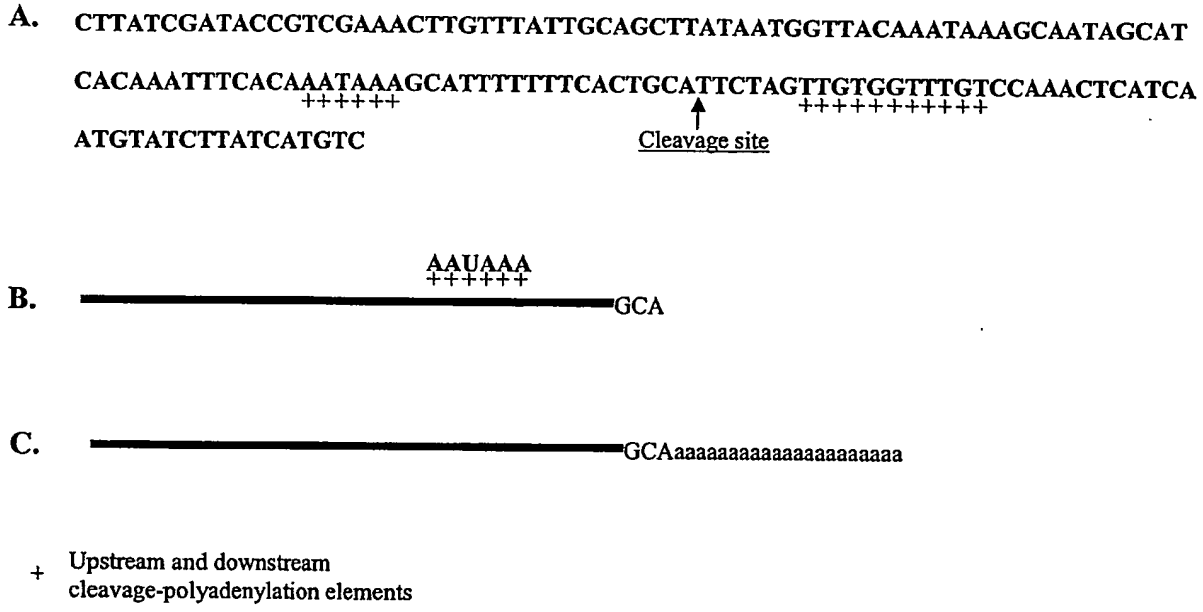


Fig 2 E1A transcription control region

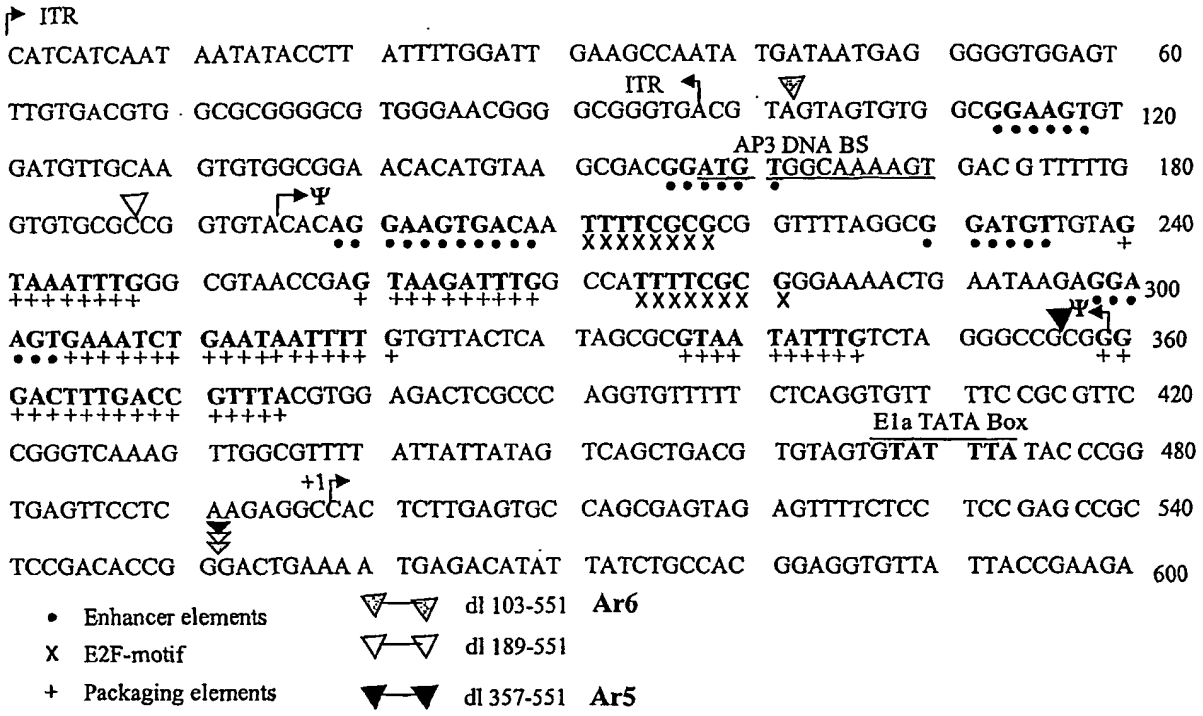


Figure 3. Sequence of Ar6pAE2fF from left and right ends of viral DNA

A. Nucleotides 1-1802 containing ITR, polyA, E2F-1 promoter, E1a and a portion of the E1b gene

```
   1 CATCATCAATAATATACCTTATTTTGGATTGAAGCCAATATGATAATGAGGGGGTGGAGT
     +-------------------------ITR-----------------------------

  61 TTGTGACGTGGCGCGGGGCGTGGGAACGGGGCGGGTGACGTAGGGCGCGATCAAGCTTAT
     +-------------------------ITR-----------+           +----

 121 CGATACCGTCGAAACTTGTTTATTGCAGCTTATAATGGTTACAAATAAAGCAATAGCATC
     ------------------------------polyA---------------------------

 181 ACAAATTTCACAAATAAAGCATTTTTTTCACTGCATTCTAGTTGTGGTTTGTCCAAACTC
     ---------------------------polyA----------------------------

 241 ATCAATGTATCTTATCATGTCTGGATCCGCGCCGCTAGCGATCATCCGGACAAAGCCTGC
     --------------------+                      +-----------------

 301 GCGCGCCCCGCCCCGCCATTGGCCGTACCGCCCCGCGCCGCCGCCCCATCTCGCCCCTCG
     ----------------------E2F-1 promoter----------------------

 361 CCGCCGGGTCCGGCGCGTTAAAGCCAATAGGAACCGCCGCCGTTGTTCCCGTCACGGCCG
     ----------------------E2F-1 promoter----------------------

 421 GGGCAGCCAATTGTGGCGGCGCTCGGCGGCTCGTGGCTCTTTCGCGGCAAAAAGGATTTG
     ----------------------E2f-1 promoter----------------------

 481 GCGCGTAAAAGTGGCCGGGACTTTGCAGGCAGCGGCGGCCGGGGGCGGAGCGGGATCGAG
     ----------------------E2f-1 promoter----------------------

 541 CCCTCGATGATATCAGATCATCGGATCCCGGTCGACTGAAAATGAGACATATTATCTGCC
     -------------+                    +--------------------------

 601 ACGGAGGTGTTATTACCGAAGAAATGGCCGCCAGTCTTTTGGACCAGCTGATCGAAGAGG
     -------------------------E1a gene---------------------------

 661 TACTGGCTGATAATCTTCCACCTCCTAGCCATTTTGAACCACCTACCCTTCACGAACTGT
     -------------------------E1a gene---------------------------

 721 ATGATTTAGACGTGACGGCCCCCGAAGATCCCAACGAGGAGGCGGTTTCGCAGATTTTTC
     -------------------------E1a gene---------------------------

 781 CCGACTCTGTAATGTTGGCGGTGCAGGAAGGGATTGACTTACTCACTTTTCCGCCGGCGC
     -------------------------E1a gene---------------------------

 841 CCGGTTCTCCGGAGCCGCCTCACCTTTCCCGGCAGCCCGAGCAGCCGGAGCAGAGAGCCT
     -------------------------E1a gene---------------------------
```

```
901  TGGGTCCGGTTTCTATGCCAAACCTTGTACCGGAGGTGATCGATCTTACCTGCCACGAGG
     -------------------------E1a gene--------------------------

961  CTGGCTTTCCACCCAGTGACGACGAGGATGAAGAGGGTGAGGAGTTTGTGTTAGATTATG
     -------------------------E1a gene--------------------------

1021 TGGAGCACCCCGGGCACGGTTGCAGGTCTTGTCATTATCACCGGAGGAATACGGGGGACC
     -------------------------E1a gene--------------------------

1081 CAGATATTATGTGTTCGCTTTGCTATATGAGGACCTGTGGCATGTTTGTCTACAGTAAGT
     -------------------------E1a gene--------------------------

1141 GAAAATTATGGGCAGTGGGTGATAGAGTGGTGGGTTTGGTGTGGTAATTTTTTTTTTAAT
     -------------------------E1a gene--------------------------

1201 TTTTACAGTTTTGTGGTTTAAAGAATTTTGTATTGTGATTTTTTTAAAAGGTCCTGTGTC
     -------------------------E1a gene--------------------------

1261 TGAACCTGAGCCTGAGCCCGAGCCAGAACCGGAGCCTGCAAGACCTACCCGCCGTCCTAA
     -------------------------E1a gene--------------------------

1321 AATGGCGCCTGCTATCCTGAGACGCCCGACATCACCTGTGTCTAGAGAATGCAATAGTAG
     -------------------------E1a gene--------------------------

1381 TACGGATAGCTGTGACTCCGGTCCTTCTAACACACCTCCTGAGATACACCCGGTGGTCCC
     -------------------------E1a gene--------------------------

1441 GCTGTGCCCCATTAAACCAGTTGCCGTGAGAGTTGGTGGGCGTCGCCAGGCTGTGGAATG
     -------------------------E1a gene--------------------------

1501 TATCGAGGACTTGCTTAACGAGCCTGGGCAACCTTTGGACTTGAGCTGTAAACGCCCCAG
     -------------------------E1a gene--------------------------

1561 GCCATAAGGTGTAAACCTGTGATTGCGTGTGTGGTTAACGCCTTTGTTTGCTGAATGAGT
     -------------------------E1a gene--------------------------

1621 TGATGTAAGTTTAATAAAGGGTGAGATAATGTTTAACTTGCATGGCGTGTTAAATGGGGC
     -------------------------+------------------------------

1681 GGGGCTTAAAGGGTATATAATGCGCCGTGGGCTAATCTTGGTTACATCTGACCTCATGGA
     -------------------------E1b gene--------------------------

1741 GGCTTGGGAGTGTTTGGAAGATTTTTCTGCTGTGCGTAACTTGCTGGAACAGAGCTCTAA
     -------------------------E1b gene--------------------------

1801 CA
     --
```

B. Nucleotides 33881-34412 containing packaging signal and ITR

```
33881 AACCTACGCCCAGAAACGAAAGCCAAAAAACCCACAACTTCCTCAAATCGTCACTTCCGT

33941 TTTCCCACGTTACGTCACTTCCCATTTTAATTAAGAATTCTACAATTCCCAACACATACA

34001 AGTTACTCCGCCCTAAAACCCTGGGCGAGTCTCCACGTAAACGGTCAAAGTCCCCGCGGC
                         +-packaging signal----------------------

34061 CCTAGACAAATATTACGCGCTATGAGTAACACAAAATTATTCAGATTTCACTTCCTCTTA
      ----------------------packaging signal----------------------

34121 TTCAGTTTTCCCGCGAAAATGGCCAAATCTTACTCGGTTACGCCCAAATTTACTACAACA
      ----------------------packaging signal----------------------

34181 TCCGCCTAAAACCGCGCGAAAATTGTCACTTCCTGTGTACACCGGCGCACACCAAAAACG
      ------------------------------------+

34241 TCACTTTTGCCACATCCGTCGCTTACATGTGTTCCGCCACACTTGCAACATCACACTTCC

34301 GCCACACTACTACGTCACCCGCCCCGTTCCCACGCCCCGCGCCACGTCACAAACTCCACC
               +-----------------ITR-----------------------------

34361 CCCTCATTATCATATTGGCTTCAATCCAAAATAAGGTATATTATTGATGATG
      --------------------------ITR---------------------+
```

Figure 4. Sequence of Ar6F from left end of viral DNA

```
  1 CATCATCAATAATATACCTTATTTTGGATTGAAGCCAATATGATAATGAGGGGGTGGAGT
    +-------------------------ITR-----------------------------------

 61 TTGTGACGTGGCGCGGGGCGTGGGAACGGGGCGGGTGACGTAGGGCGCGCCGCTAGCGAT
    ---------------------------ITR---------------++------MCS--------

121 ATCGGATCCCGGTCGACTGAAAATGAGACATATTATCTGCCACGGAGGTGTTATTACCGA
    ---------------+------------E1a-------------------------------

181 AGAAATGGCCGCCAGTCTTTTGGACCAGCTGATCGAAGAGGTACTGGCTGATAATCTTCC
    ----------------------------E1a-------------------------------

241 ACCTCCTAGCCATTTTGAACCACCTACCCTTCACGAACTGTATGATTTAGACGTGACGGC
    ----------------------------E1a-------------------------------

301 CCCCGAAGATCCCAACGAGGAGGCGGTTTCGCAGATTTTTCCCGACTCTGTAATGTTGGC
    ----------------------------E1a-------------------------------

361 GGTGCAGGAAGGGATTGACTTACTCACTTTTCCGCCGGCGCCCGGTTCTCCGGAGCCGCC
    ----------------------------E1a-------------------------------

421 TCACCTTTCCCGGCAGCCCGAGCAGCCGGAGCAGAGAGCCTTGGGTCCGGTTTCTATGCC
    ----------------------------E1a-------------------------------

481 AAACCTTGTACCGGAGGTGATCGATCTTACCTGCCACGAGGCTGGCTTTCCACCCAGTGA
    ----------------------------E1a-------------------------------

541 CGACGAGGATGAAGAGGGTGAGGAGTTTGTGTTAGATTATGTGGAGCACCCCGGGCACGG
    ----------------------------E1a-------------------------------

601 TTGCAGGTCTTGTCATTATCACCGGAGGAATACGGGGGACCCAGATATTATGTGTTCGCT
    ----------------------------E1a-------------------------------
```

Figure 5. Sequence of Ar6pAF from left end of viral DNA

```
   1 CATCATCAATAATATACCTTATTTTGGATTGAAGCCAATATGATAATGAGGGGGTGGAGT
     +--------------------------ITR-----------------------------------

  61 TTGTGACGTGGCGCGGGGCGTGGGAACGGGGCGGGTGACGTAGGGCGCGATCAAGCTTAT
     ---------------------------ITR--------------+             +----

 121 CGATACCGTCGAAACTTGTTTATTGCAGCTTATAATGGTTACAAATAAAGCAATAGCATC
     --------------------------polyA-------------------------------

 181 ACAAATTTCACAAATAAAGCATTTTTTTCACTGCATTCTAGTTGTGGTTTGTCCAAACTC
     -------------------------polyA-------------------------------

 241 ATCAATGTATCTTATCATGTCTGGATCCGCGCCGCTAGCGATATCGGATCCCGGTCGACT
     --------------------+                                       +--

 301 GAAAATGAGACATATTATCTGCCACGGAGGTGTTATTACCGAAGAAATGGCCGCCAGTCT
     --------------------------E1a--------------------------------

 361 TTTGGACCAGCTGATCGAAGAGGTACTGGCTGATAATCTTCCACCTCCTAGCCATTTTGA
     --------------------------E1a--------------------------------

 421 ACCACCTACCCTTCACGAACTGTATGATTTAGACGTGACGGCCCCCGAAGATCCCAACGA
     --------------------------E1a--------------------------------

 481 GGAGGCGGTTTCGCAGATTTTTCCCGACTCTGTAATGTTGGCGGTGCAGGAAGGGATTGA
     --------------------------E1a--------------------------------

 541 CTTACTCACTTTTCCGCCGGCGCCCGGTTCTCCGGAGCCGCCTCACCTTTCCCGGCAGCC
     --------------------------E1a--------------------------------

 601 CGAGCAGCCGGAGCAGAGAGCCTTGGGTCCGGTTTCTATGCCAAACCTTGTACCGGAGGT
     --------------------------E1a--------------------------------
```

Figure 6. Schematic diagram of Ar6pAF and Ar6pAE2fF vectors

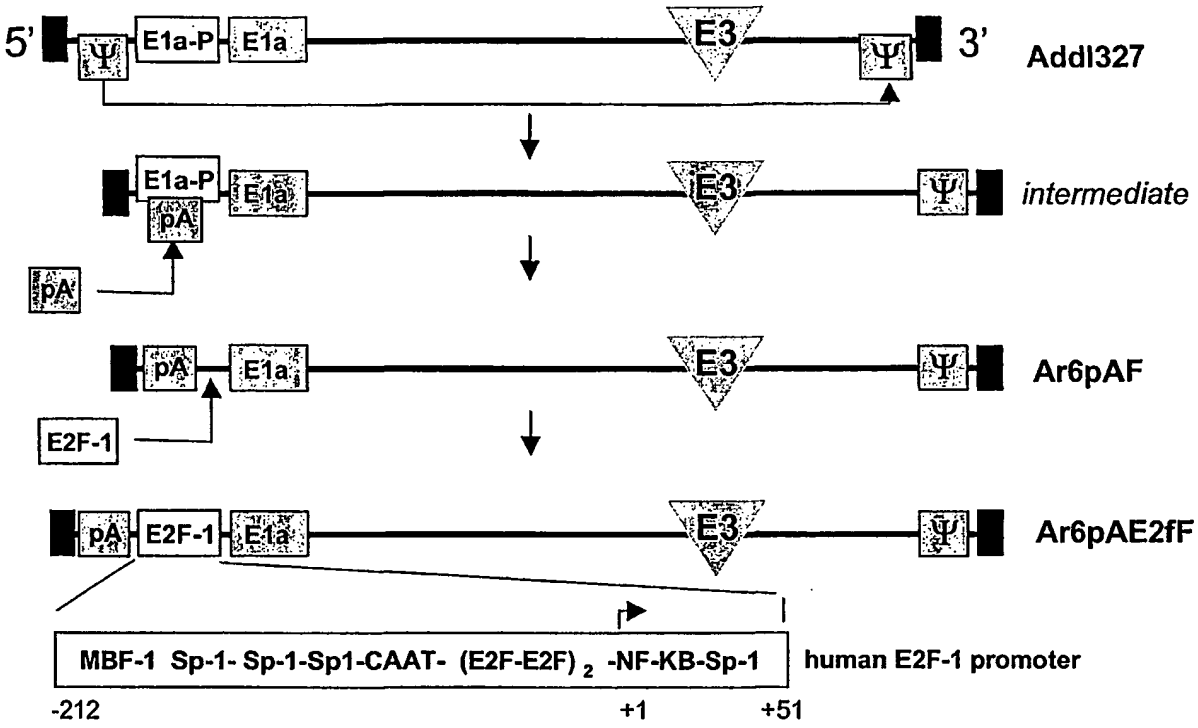


Fig. 7 Body weight change

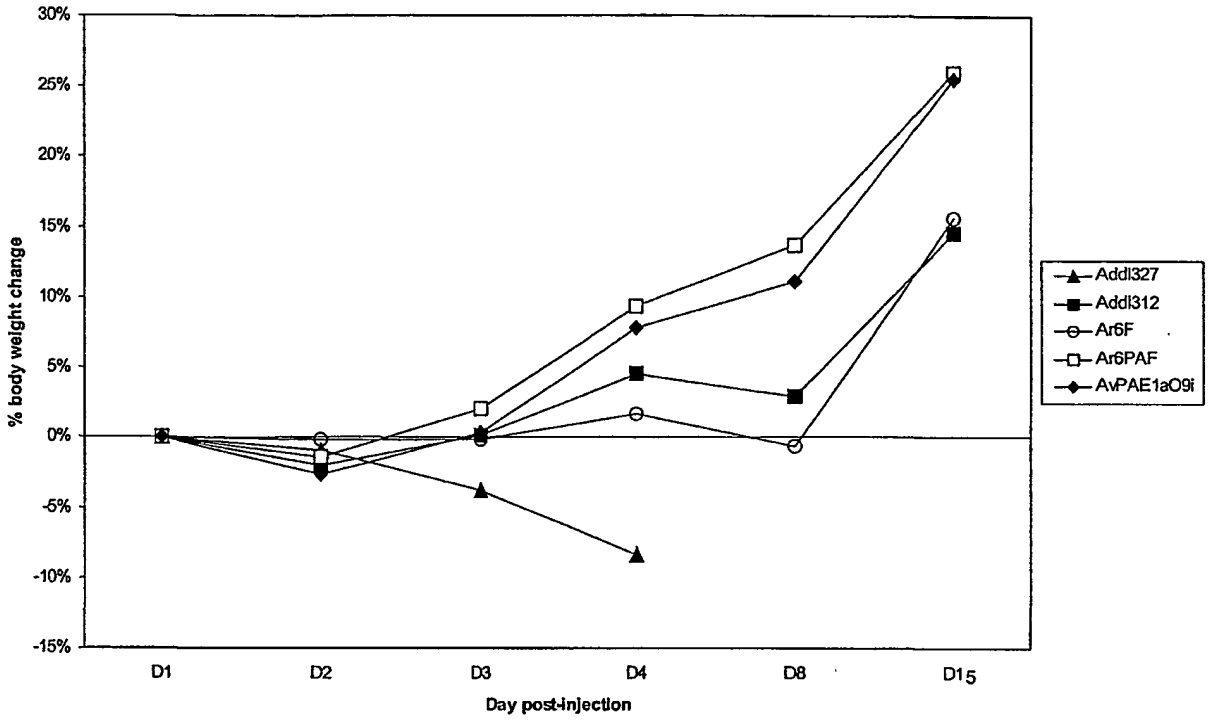


Fig. 8 Minimizing nonspecific transactivation of E1a gene

Backbones generated: